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Estrogen Receptor Factor-1 (ERF-1) is a transcription factor present in estrogen receptor (ER)-positive breast and endometrial carcinoma cell lines that binds to the untranslated leader sequence of ER, thus inferring a role for this protein in the transcriptional regulation of ER. There are two binding sites for ERF-1 in the untranslated leader sequence; a high affinity distal site and a lower affinity proximal site. The minimal ERF-1 binding site was mapped using double point mutants in competitive gel shift assays. The molecular weight of ERF-1 was determined using gel renaturation with competitors and UV crosslinking techniques. A protein purification strategy using ion-exchange and DNA affinity chromatography was developed to purify ERF-1 from MCF7 human breast carcinoma cells. Purified ERF-1 is currently being microsequenced and the sequence information will be used to clone the cDNA for ERF-1 from a MCF7 expression library.

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INTRODUCTION

Background

The expression of estrogen receptor (ER) is intimately associated with the biology of breast carcinoma. Breast carcinomas occurring in postmenopausal women are often ER-positive [1] and many of these tumors express significantly more receptor than normal mammary epithelium [2]. ER-negative breast carcinomas are more likely to occur in young women and these tumors carry a worse prognosis than carcinomas which express ER [3, 4]. Several studies have focused on the function of ER in an attempt to explain the association between ER expression and tumor biology. Mutations have been described in the ER gene of some breast carcinomas that render these altered ER proteins incapable of binding estrogen response elements (ERE) [5, 6] and able to inhibit wild-type ER function [7, 8]. Other studies, however, have found ER mutations which result in a constitutively active receptor which has also been postulated as important to the development of hormone-independent growth [9, 10]. If ER function is influencing the oncogenic process, it is difficult to conceptualize within a single model of oncogenesis the occurrence of mutations which inhibit ER function and mutations which result in constitutive activity. An alternative hypothesis is that mechanisms regulating transcription of the ER gene influence the phenotype of breast carcinoma; within this model, ER-negative carcinomas which do not transcribe the ER gene define a subset of tumors with a more aggressive phenotype. This theory is supported by recent studies which have identified breast carcinoma cell lines that fail to transcribe an apparently normal ER gene [11]. It is therefore possible that defining molecular mechanisms controlling transcription of the ER gene may provide new insight into the biology of breast carcinoma.

Transcription of ER occurs from two separate promoters, P0 and P1 [12], although no functional mapping has been previously published. P1 is the major ER transcriptional start site [13] and is predominantly utilized in human mammary epithelial cells (HMEC) and is the major start site in ER-positive human breast carcinomas [14]. Multiple cap sites have been identified for the P0 promoter. Studies of the murine ER gene identified 10 cap sites spanning approximately 60 bases [15] and a start site at -1994 (from the P1 cap site) was identified in human cells which would agree closely with the major murine P0 cap site [16]. Transcription from the P0 promoter is characteristic of human endometrial tissue and can account for 12 to 33% of ER transcription in breast carcinoma cells [14]. Recently, several novel transcription start sites have been identified, including two, P_E and P_H, which splice into +163 of the untranslated leader sequence as does the P0 transcript [17]. In transcript P_E, the sequence upstream from the +163 splice site corresponds to -359 to -169 (relative to the P1 start site), a region previously described as part of the intron between P0 and P1. The P_H cap site has been shown to be at least 20 kb upstream of the P1 promoter, suggesting the existence of an alternate promoter for ER.

Recently, a transcription factor, Estrogen Receptor Factor-1 (ERF-1), was identified that binds to the untranslated leader sequence of the ER gene [18]. Two ERF-1 binding sites were identified in this region by mutational analysis. The distal binding site, located from +182 to +201, has a higher affinity for ERF-1 binding than the proximal binding site, located from +132 to +171. ERF-1 is present in ER-positive breast and endometrial carcinoma cells, but absent in ER-negative cell lines. This infers a role for ERF-1 in the regulation of ER transcription in breast carcinoma.

Purpose of Present Work

These experiments are designed to investigate mechanisms regulating transcription of the ER gene and to characterize the ER transcription factor, ERF-1. The experiments described address technical objective #3 of this U.S. Army Research Grant DAMD17-94-J-4353. The ERF-1 binding site is defined and precisely mapped using mutational analysis and competitive gel shift assays. Renaturation and UV crosslinking experiments are performed to determine the molecular weight of ERF-1 and the protein is purified using ion-exchange and sequence specific DNA affinity chromatography for protein microsequencing. Sequence information will be used to clone the cDNA for ERF-1.

BODY

Methods of Approach

Cell lines. The MCF7 ER-positive human breast carcinoma cell line was obtained from American Type culture Collection, Rockville, MD. Cells were maintained in minimal essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine sera (Hyclone, Logan, UT), 25 mM 4-(2-hydroxyethey)-1piperazineethanesulfonic acid (Hepes), 26 mM sodium bicarbonate, 5,000 units/ml penicillin G (Gibco BRL), 5,000 ug/ml streptomycin (Gibco BRL), and 6 ng/ml bovine insulin (Sigma Chemical Company, St. Louis, MO). Cells were incubated at 37°C in 5% CO_2.

Harvesting MCF7 nuclei. MCF7 cells were collected by trypsinization and pelleted at 2,830 x g at 4° C for 10 minutes. Cell pellets were washed in ice-cold 1X Phosphate Buffered Saline (PBS) and repelleted as above. After removal of the supernatant, the packed cellular volume (PCV) was determined. The cells were resuspended in 5 times PCV of Buffer A (100 mM Hepes (pH 7.9), 100 mM magnesium chloride ($MgCl_2$), 400 mM KCl, 1 mM dithiothreitol (DTT), 1 mM sodium metabisulfite (NaMetaBis), 0.2 mM phenylmethylsulfonyl (PMSF)) and allowed to swell on ice for 20 minutes followed by centrifugation at 2,960 x g at 4° C for 10 minutes. The cell pellet was resuspended in 2 times PCV in Buffer A and the cell suspension was homogenized in a

Dounce homogenizer with 9 up-and-down strokes using pestle B. The nuclei were pelleted at 1,450 x g at 4° C for 10 minutes and were stored at -80°C.

Preparation of MCF-7 nuclear extract. MCF7 nuclei were pooled and the packed nuclear volume (PNV) was determined. Nuclei were resuspended in 0.6 times PNV of buffer D (10 mM Tris pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM NaMetaBis, 0.2 mM PMSF) and dounce homogenized on ice with 10 strokes. Nuclei were mixed with 0.06 times PNV of 4 M ammonium sulfate, pH 7.9 and extracted with gentle mixing at 4° C for 1 hour. Extracted nuclei were pelleted by centrifugation at 25,000 x g, 4° C for 20 minutes. Nuclear extract was transferred to dialysis tubing and dialyzed for 5.5 hours against 5 liters of D-100 buffer (20 mM Hepes pH 7.9, 20% glycerol, 100 mM potassium chloride (KCl), 2 mM $MgCl_2$, 0.2 mM ethylenedinitrilotetraacetic acid (EDTA), 1 mM DTT, 1 mM NaMetaBis, 0.2 mM PMSF). The extract was removed from the dialysis tubing and centrifuged for 20 minutes at 16,000 x g, 4°C to remove particulate matter. Protein concentration was determined using a Bio-Rad protein assay and generally fell within the 7-15 mg/ml range. Extract was stored at -80° C until use.

Gel Shift Assay. Ten to fifteen micrograms of nuclear cell extract or purified chromatographic fractions were incubated in 1x binding buffer (40 mM KCl, 20 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM DTT), 4% Ficoll, 12 ug/ml poly dI-dC (Pharmacia), 1 mg/ml Bovine Serum Albumin (BSA) (Sigma) and 0.2 ng of radiolabelled double-stranded oligonucleotide probe in a volume of 25 ul at room temperature for 1 hour. In competitive binding assays, unlabelled double-stranded oligonucleotides containing point mutations were added at an 1,000 fold excess. Reactions were then loaded on a 4% acrylamide gel in 0.25X TBE and electrophoresed at 270 volts at 4°C. Gels were dried and exposed to x-ray film.

Double-stranded oligonucleotides were prepared by synthesizing each strand separately, then mixing equimolar ratios of each in 0.3 M sodium acetate and boiling for 10 minutes. The mixture was allowed to cool slowly to room temperature and the double-stranded oligonucleotides were then ethanol precipitated and resuspended in water at a concentration of 200 ng/ml. Probe was prepared by end-labeling double stranded oligonucleotides with $\gamma\,^{32}P$ Adenosine triphosphate (3,000 Ci/mmol) using T4 polynucleotide kinase in an exchange reaction, followed by gel purification. Twenty or thirty base pair oligonucleotides used as competitors were synthesized with the following double point mutations (underlined):

imperfect palindrome

WT: 5' TGAGCCTTCTG<u>CCCTGCGGGG</u>ACACGGTCT 3'

mutant #1: 5' <u>AC</u>AGCCTTCTGCCCTGCGGGGACACGGTCT 3'

mutant #2: 5' TGTCCCTTCTGCCCTGCGGGGACACGGTCT 3'

mutant #3: 5' GGTTCTGCCCTGCGGGGACA 3'

mutant #4: 5' CCAACTGCCCTGCGGGGACA 3'

mutant #5: 5' CCTTGAGCCCTGCGGGGACA 3'

mutant #6: 5' CCTTCTCGCCTGCGGGGACA 3'

mutant #7: 5' CCTTCTGCGGTGCGGGGACA 3'

mutant #8: 5' CCTTCTGCCCACCGGGGACA 3'

mutant #9: 5' CCTTCTGCCCTGGCGGGACA 3'

mutant #10: 5' CCTTCTGCCCTGCGCCGACA 3'

mutant #11: 5' CCTTCTGCCCTGCGGGCTCA 3'

mutant #12: 5' CCTTCTGCCCTGCGGGGAGT 3'

mutant #13: 5' TGAGCCTTCTGCCCTGCGGGGACAGCGTCT 3'

mutant #14: 5' TGAGCCTTCTGCCCTGCGGGGACACGCACT 3'

mutant #15: 5' TGAGCCTTCTGCCCTGCGGGGACACGGTGA 3'

Renaturation of ERF-1 activity from SDS-PAGE . Two milligrams of MCF7 nuclear extract was resolved on 8% SDS-PAGE after which the gel was segmented into sequential 4 mm slices ranging from 31 to 220 kDa. Each slice was transferred to an eppendorf tube and crushed in 0.4 ml elution buffer (0.15M sodium chloride (NaCl), 0.1% sodium dodecyl sulfate (SDS), 0.05 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 5 mM DTT, 0.1 mg/ml BSA) with a teflon pestle. Protein was eluted for 1 hour at room temperature, the crushed gel was pelleted and the supernatant removed to a new tube. Protein was precipitated with 4 volumes of cold acetone and recovered by

centrifugation at 12,000 x g for 10 minutes. Residual SDS was removed by washing with cold 80% acetone. Precipitated protein was dissolved in 0.5 ml of 8M Urea in D-100 buffer and allowed to denature at 4°C for 30 minutes with gentle rocking. The protein solution was transferred to dialysis tubing and dialyzed against 1 liter of 1M urea in D-100 buffer for 3 hours, followed by dialysis against 2 changes of D-100 buffer alone for 3 hours and 12 hours respectively. The protein solution was then removed from the dialysis tubing and quick-frozen in liquid N_2 for storage at -80°C until subsequent use in gel shift assays.

UV crosslinking of ERF-1. Ten to fifteen micrograms of nuclear cell extract or purified chromatographic fractions were incubated in 1x binding buffer (50 mM KCl, 20 mM Hepes, pH 7.9, 5% glycerol, 1 mM EDTA, 1mM DTT) with 0.1 mg/ml poly dI-dC, 0.1 mg/ml p(dN_6) (Pharmacia), 0.1 mg/ml BSA and 1 x 10^6 cpm of radiolabelled double-stranded oligonucleotide probe in a volume of 20 ul at room temperature for 35 minutes. Ficoll was added to 3% and the reactions were loaded on a 4% acrylamide gel in 0.25X TBE and electrophoresed at 270 volts at 4°C. The gel was wrapped in saran wrap and exposed to 305 nm UV irradiation for 10 minutes to crosslink the ERF-1 complexes. The wet film was then exposed to x-ray film for 10-16 hours at 4°C to visualize the DNA-protein complexes. After development, the film was aligned with the gel and the crosslinked ERF-1 complexes were excised and placed in eppendorf tubes. Fifty microliters of 2X SDS loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue (BPB), 20% glycerol) containing 5% 2-mercaptoethanol and 6M urea was added to each gel slice and boiled for 10 minutes. The gel slices were then inserted into the wells of an 1.6 mm thick 8% SDS-PAGE gel with a spatula and overlayed with the 50 ul of loading dye. The samples were elecrophoresed with pre-stained molecular weight markers (Amersham, Arlington Heights, IL) at 225 volts at room temperature until the BPB ran off the bottom. The gel was then dried and exposed to x-ray film.

Radiolabelled probe was prepared by mixing 1 pmole of an antisense 27-mer oligonucleotide encompassing the ERF-1 binding site (5' CCGTGTCCCCGCAGGGCAGAAGGCTCA 3') (Operon Technologies, Alameda, Ca) with 100 pmole of an oligonucleotide (5' TGAGCCTTCT 3') (Ana-Gen Technologies, Palo Alto, Ca) complimentary to the 3' terminal 10 bases. The reaction was carried out in a volume of 5 ul at 50 mM NaCl . The oligonucleotide mixture was heated to 80°C for 3 minutes, and allowed to anneal with slow cooling over one hour. The annealed template/primer complex was then added to 20 ul nucleotide/enzyme mixture resulting in a final concentration of 4 mM dATP (Pharmacia), 4 mM BrdU-5-bromo-2'-deoxyuridine 5'-triphosphate, Na+ salt (BrdU; Sigma), 50 µCi α-^{32}P dCTP (3,000 Ci/mmole, Amersham), 50 µCi α-^{32}P dGTP (3,000 Ci/mmole, Amersham), 10mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithioerythritol and 5.9 units Klenow fragment

of DNA polymerase I (Pharmacia). After incubation at 16°C for 2 hours, the reaction was chased with 1mM each dNTP and an additional 5.9 units Klenow fragment and incubated at 16°C for a further 30 minutes. The probe was then extracted with phenol and passed over a Biospin 6 column (Biorad, Hercules, CA) to remove free ^{32}P. The desalted probe was adjusted to 0.3M sodium acetate and 20 ug tRNA (Pharmacia) was added prior to precipitation with 2 volumes of 100% ethanol. The probe was recovered by centrifugation at 13,000 x g for 10 minutes at 4°C and the pellet was washed with 70% ethanol and then resuspended in 20 ul TE buffer. Typically, 2 x 10^7 cpm was incorporated using 1 pmol of starting template.

Column Chromatography of ERF-1. Three hundred and seventy-five milligrams of MCF7 nuclear extract was applied to a 30 ml Q Sepharose Fast Flow anion exchange column (Pharmacia) at 100mM KCl and ERF-1 activity, as monitored by gel shift assay, was recovered in the flow-through fraction. This fraction was applied to a 10 ml Heparin Sepharose CL-6B column (Pharmacia), washed with 50 mls of 0.35M KCl and then ERF-1 was eluted with 25 mls of 0.6M KCl. Two fractions were collected with ERF-1 eluting in the last 20 mls. This 0.6M fraction was diluted to 0.1M KCl, applied to a 5 ml DNA cellulose column (native DNA, Pharmacia), and eluted with 12.5 mls of 0.4M KCl of which the last 10 mls contained ERF-1. This fraction was diluted to 0.15M KCl and a 30-mer ERF-1 binding site mutant (5' TGAGCCTTCTGCGGTGCGGGGACACGGTCT 3') that had previously been shown not to bind ERF-1 was added at 10 ug/ml, incubated on ice 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C to clear any precipitated material. This fraction was then split in two and each half was loaded onto an 1 ml DNA affinity column. The columns were washed with 5 mls of 0.5M KCl and then ERF-1 was eluted with 5 mls of 0.8M KCl. Five fractions of 0.5 ml, 1.5 ml, 0.5 ml, 1.25 ml and 1.25 ml were collected. The DNA affinity column was prepared by attaching a double-stranded, biotinylated 30-mer oligonucleotide (5' TGAGCCTTCTGCCCTGCGGGGACACGGTCT 3') corresponding to the wild-type ERF-1 binding site to streptavidin agarose. All fractions were quick frozen in liquid nitrogen and stored at -80°C.

Gradient Elution of ERF-1. Chromatography was performed as described above except that only 75 mg of nuclear extract was used and the columns were reduced to a 25 ml Q Sepharose column, a 5 ml Heparin Sepharose column, an 1 ml DNA cellulose column and a single 1 ml DNA affinity column. In addition, the 0.5M to 0.8M KCl step elution from the DNA affinity column was replaced with a 10 ml gradient elution from 0.35M to 0.8M KCl in which 1 ml fractions were collected.

TCA Precipitation and SDS-PAGE Analysis of ERF-1. Protein fractions were concentrated by precipitation with 6% tricholoroacetic acid (TCA) in the presence of 0.014% deoxycholic acid. Protein was recovered by centrifugation in round bottom 12 x 75 mm disposable test tubes at 3,000 x g for 15 minutes. Protein pellets were washed with 80% acetone, resuspended in standard Laemmli 1 x SDS loading buffer, boiled 3 minutes and electrophoresed on 8% SDS-PAGE with standard protein molecular weight markers. Proteins were visualized using the Silver Stain Plus kit (Bio-Rad).

Preparation of ERF-1 for Protein Microsequencing. Approximately forty pmoles of ERF-1 was TCA precipitated from the 0.8M KCl DNA affinity fraction and resuspended in 1 x SDS loading buffer as described above. After resolution on 8% SDS-PAGE, the gel was stained briefly with Coomassie Brillant Blue and destained thoroughly in 3 changes of destain solution (5% methanol, 7% acetic acid) over 1.5 hours until the background was clear. The gel was rinsed briefly in water and the ERF-1 band was excised along with an equivalent piece of gel from the neighboring empty lane for use as a background control. The gel slices were placed in separate tubes and washed with 50% acetonitrile with gentle rocking for 5 minutes at room temperature. The supernatant was removed and the wash was repeated one more time. The slightly moist gel slices were frozen and shipped on dry ice to the Harvard Microchemistry Facility for tryptic digestion and subsequent internal peptide sequencing.

RESULTS

Mapping of the ERF-1 Distal Binding Site

Previously, it had been shown that the untranslated leader sequence of the estrogen receptor contained a distal, high affinity binding site for ERF-1 within the sequences +182 to +201 and a second, lower affinity proximal binding site from +132 to +171 [18]. In these original experiments, an 80 bp probe containing both the proximal and distal binding sites was used to define ERF-1 activity in various carcinoma cell lines. To elucidate the minimal sequence needed for ERF-1 binding, gel shift assays were performed using probes containing various combinations of the distal binding site. A single site 30 bp probe (5' TGAGCCTTCTGCCCTGCGGGGACACGGTCT 3'), which corresponds to the distal site flanked by 10 bp, was able to bind ERF-1 as efficiently as the full-length 80 bp probe. A 20 bp probe (5' CCTTCTGCCCTGCGGGGACA 3'), containing only the core distal sequence from +182 to +201, bound ERF-1 with slightly less affinity. A double site 45 bp probe (5' CCTTCTGCCCTGCGGGGACACGGTCCCTTCTGCCCTGCGGGGACA 3'), consisting of two distal sites separated by a 5 bp spacer also bound ERF-1 as well as the 80 bp probe and shows that there is a sequential binding to the second site with increasing amount of cellular extract.

There does not appear to be a facilitation of binding to the second site upon occupation of the first site.

More precise mapping of the distal site was done by gel shift competition using oligonucleotides containing double point mutations within the sequences +178 to +207. The oligonucleotides were either 20 bp sequences containing only the distal site or 30 bp sequences containing this site plus several flanking bases. Mutations in the flanking sequences (mutants 1,2,13-15) did not have any effect on ERF-1 binding, while changes within the core distal site (mutants 3-12) destroyed binding to ERF-1 to differing degrees, ranging from 50 to 100%. Mutations which disrupted the 10 bp imperfect palindrome (CCCTGCGGGG; mutants 7-10) contained within the high affinity site obliterated ERF-1 binding completely. These data indicate that integrity of the imperfect palindrome is important in maintaining ERF-1 binding.

Renaturation of ERF-1

To determine the molecular weight of the ERF-1 specific binding protein, MCF7 nuclear protein was resolved on SDS-PAGE and renatured from individual gel slices. Nine gel slices ranging in size from 31 to 220 kDa were excised and the renatured protein fractions were tested for ERF-1 binding activity by gel shift assay. The 30 bp wild type ERF-1 binding site oligonucleotide was used as a probe and MCF7 nuclear extract that had not been denatured was used in the assay for comparison. This technique identified approximately 5 different DNA-protein complexes in various fractions. Complexes formed in fractions 4, 5 and 6 migrated similarly to the ERF-1 complex found in the non-denatured MCF7 nuclear extract. To confirm if any of these complexes was indeed ERF-1, competitive gel shift assays were performed with a panel of binding site mutants and compared to the native gel shift pattern. The complex in fraction 4 was competed by the mutant competitors in an identical fashion as native ERF-1, indicating that ERF-1 was contained in this fraction. The DNA-protein complexes found in fractions 5 and 6 showed a different pattern of competition. Fraction 4 correlates to a size range of approximately 50 to 60 kDa.

UV Crosslinking of ERF-1

UV crosslinking was used as an additional method to confirm the molecular weight of ERF-1. ERF-1 complexes were formed in solution using MCF7 nuclear extract as well as increasingly more purified chromatographic fractions from Q sepharose, Heparin Sepharose and DNA cellulose columns. After resolution on a non-denaturing acrylamide gel, the complexes were UV irradiated to irreversibly bind ERF-1 to the probe. The probe contained a BrdU residue in place of thymidine in the ERF-1 binding site to increase the ability of the DNA to crosslink to ERF-1. After the complexes were subjected to SDS-PAGE, autoradiography revealed a band in each

sample that migrated at approximately 60 to 66 kDa. This indicates that ERF-1 has an approximate molecular weight of 51-57 kDa when the 8-9 kDa of the bound strand of crosslinked probe is taken into account. This agrees well with the size determined by the protein renaturation experiments above.

Column Chromatography and Gradient Elution of ERF-1

As an initial step in the purification of ERF-1, nuclear extract prepared from MCF7 breast carcinoma cells was tested on several ion exchange chromatography matrices to determine its binding characteristics. The three matrices chosen were Q sepharose Fast Flow (anion exchanger), S sepharose Fast Flow (cation exchanger) and Heparin sepharose CL-6B (cation/DNA affinity). MCF7 nuclear extract was applied to 1 ml columns and ERF-1 activity was eluted with increasing KCl concentration. ERF-1 activity was recovered in the flow-through from the Q sepharose column and was eluted at 0.6M KCl from both the S sepharose and Heparin sepharose columns, thus indicating that ERF-1 is a positively charged protein. From these preliminary experiments, a chromatographic purification protocol was designed combining the ion exchange capabilities of Q and Heparin sepharose and the DNA affinity properties of native DNA-cellulose and ERF-1 specific affinity chromatography. Purification of ERF-1 was monitored by gel shift assay and by SDS-PAGE to visualize proteins remaining in active fractions. Using this protocol, several bands in the appropriate size range were apparent in the active fraction eluted from ERF-1 specific affinity chromatography.

To determine which of these proteins corresponded to ERF-1, a small-scale purification was performed in which the final step elution from the ERF-1 specific DNA affinity column was replaced with a gradient elution. A gradient elution allows for a finer separation of proteins within a fraction than does the step elution, thus facilitating the correlation between ERF-1 activity and a specific protein band on a SDS-PAGE gel. Using this method, an approximately 50 kDa protein band was identified as ERF-1.

Purification of ERF-1 for Protein Microsequencing

From initial experiments, it was determined that a minimum of 1.5×10^{11} MCF7 cells were needed to purify enough ERF-1 for tryptic digestion and internal peptide sequencing. Nuclei were harvested and approximately 2.5 grams of nuclear extract was prepared from 2×10^{11} cells. ERF-1 was purified sequentially over Q sepharose, Heparin sepharose, DNA cellulose and ERF-1 specific DNA affinity chromatography in 7 batches of 375 ug each. Gel shift assays showed that the bulk of ERF-1 activity was eluted in the second 0.8M KCl DNA affinity fraction with activity tailing into fractions 3-5. Protein from these fractions was precipitated and approximately 3 ug of ERF-1 was recovered as determined by gel quantitation of a small aliquot with standard protein

molecular weight markers. The remaining ERF-1 protein was gel purified using SDS-PAGE and the excised protein band was sent to the Harvard Microchemistry Facility where it is currently being digested with trypsin and sequenced.

DISCUSSION

Although several mechanisms are involved in controlling expression of ER in breast carcinomas, transcriptional regulation is clearly responsible for the ER-negative phenotype in some cell lines [11, 19]. Previously, an important transcriptional regulatory element was identified in the 5' untranslated leader sequence of the ER gene that contained two binding sites for a transcription factor, ERF-1 [18]. ERF-1 was shown to be abundantly expressed in a panel of ER-positive breast and endometrial cell lines, while ER-negative cell lines did not express this protein. This indicates a role for ERF-1 in the transcriptional regulation of ER. Although there are likely other regulatory elements involved in the regulation of ER transcription, the identification of ERF-1 offers a molecular mechanism that accounts for differences in ER expression found in breast carcinomas.

Data derived from previous southwestern blot experiments had suggested that ERF-1 was a 30 kDa protein [18]. Although DNA affinity chromatography of nuclear extracts from MCF7 breast carcinoma cells revealed an approximate 30 kDa protein in the purified active fraction, protein microsequencing revealed this as the contaminant histone H1. Data derived from southwestern blots could be inaccurate due to non-specific interactions of probe with the blotted proteins. The histone H1 contaminant was effectively removed by the addition of a native DNA cellulose chromatography step in the purification protocol. The molecular weight of ERF-1 was redefined to be approximately 50 kDa using the two alternate methods of renaturation and UV crosslinking. Both of these techniques circumvent the problem of non-specific binding of DNA probe to proteins other than ERF-1. A prominent 50 kDa protein present in the purified active fraction after DNA affinity purification has been putatively identified as ERF-1.

CONCLUSIONS

The identification of ERF-1 offers new insight into our understanding of the relationship between ER expression and the biology of breast carcinoma. Because ER is a transcription factor, many researchers have suggested that the phenotype displayed by ER-positive breast carcinomas is due to the repertoire of genes whose expression is regulated through estrogen response elements. Alternatively, the expression of ER may be a marker for the degree of differentiation of a tumor while ERF-1, by regulating a number of cellular genes including ER, may be the factor that establishes the differentiated phenotype. Furthermore, the identification of ERF-1 has immediate clinical relevance in that tumors that lack ERF-1 expression might define a subset of cancer patients

with a prognosis different from patients with ER-negative tumors where loss of expression is due to mutations within the ER gene. Understanding the control of ERF-1 may also provide new therapeutic approaches to the treatment of aggressive ER-negative tumors.

The next objective of this research project is to use partial protein sequence obtained from internal microsequencing of purified protein to clone the cDNA for ERF-1 as described in objective 3 of this grant proposal. Purified ERF-1 protein is currently being sequenced at the Harvard Microchemistry Facility. Once an ERF-1 cDNA is obtained, its role in the regulation of ER transcriptional regulation will be examined as outlined in objective 4.

REFERENCES

1. Iglehart, J.D., *The breast,* in *Textbook of Surgery: The biological basis of modern surgical practice,* D.C. Sabiston, Editor. 1986, W. B. Saunders Company: Philadelphia. p. 510.

2. Ricketts, D., *et al., Estrogen and progesterone receptors in the normal female breast.* Can. Res., 1991. **51**: p. 1817-1822.

3. Knight, W.A., *et al., Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer.* Cancer Res., 1977. **37**: p. 4669-4671.

4. Clark, G.M., *et al., How to integrate steroid hormone receptor, flow cytometric, and other prognostic information in regard to primary breast cancer.* Cancer sup., 1993. **71**: p. 2157-2162.

5. Watts, C.K.W., *et al., Oestrogen receptor gene structure and function in breast cancer.* J. Steroid Biochem. Molec. Biol., 1992. **41**(3): p. 529-536.

6. Scott, G.K., *et al., Truncated forms of DNA-binding estrogen receptors in human breast cancer.* J. Clin. Invest., 1991. **88**: p. 700-706.

7. Ince, B.A., *et al., Powerful dominant negative mutants of the human estrogen receptor.* J. Bio. Chem., 1993. **268**(19): p. 14026-14032.

8. Fuqua, S.A.W., *et al., Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors.* Can. Res., 1992. **52**: p. 483-486.

9. McGuire, W.L., G.C. Chamness, and S.A.W. Fuqua, *Estrogen receptor variants in clinical breast cancer.* Mol. Endocr., 1991. **5**(11): p. 1571-1577.

10. Castles, C.G., *et al., Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line.* Can. Res., 1993. **53**: p. 5934-5939.

11. Weigel, R.J. and E.C. deConink, *Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma.* Cancer Res., 1993. **53**: p. 3472-3474.

12. Keaveney, M., *et al., Evidence for a previously unidentified upstream exon in the human oestrogen receptor gene.* J. Mol. Endo., 1985. **6**: p. 111-115.

13. Green, S., *et al., Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A.* Nature, 1986. **320**: p. 134-139.

14. Weigel, R.J., *et al., Quantitative analysis of the transcriptional start sites of estrogen receptor in breast carcinoma.* Cell Growth and Differentiation, 1995. **6**: p. 707-711.

15. White, R., *et al., Structural organization and expression of the mouse estrogen receptor.* Mol. Endo., 1987. **87**: p. 735-744.

16. Piva, R., *et al., Sequencing of an RNA transcript of the human estrogen receptor gene: evidence for a new transcriptional event.* J. Steroid Biochem. Molec. Biol., 1993. **46**(5): p. 531-538.

17. Thompson, D.A., *et al., Identification of two estrogen receptor transcripts with novel 5' exons isolated from a MCF7 cDNA library.* submitted for publication, 1996.

18. de Coninck, E.C., L.A. McPherson, and R.J. Weigel, *Transcriptional Regulation of Estrogen Receptor in Breast Carcinoma.* Mol. Cell. Biol., 1995. **15**(4): p. 2191-2196.

19. Ottaviano, Y.L., *et al., Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells.* Can. Res., 1994. **54**: p. 2552-2555.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Jan 00

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FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

94-J-4353 AD-B221 777

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